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Keywords: Substance P; blood-brain barrier; LC-MS/MS

Abstract: Substance P (SP) has been associated with pain, depression as well as neurodegenerative diseases. Many of these diverse actions of SP can potentially be attributed to SP metabolites generated at the blood-brain barrier (BBB). In these studies, the metabolism of SP was investigated using an *in vitro* model of the BBB and LC-MS/MS. Substance P metabolism was found to be non-saturable in the concentration range of 100 nM to 10 μ M, with approximately 70% of the peptide remaining intact after 5 hrs. The major metabolites of SP were identified by MS to be 3-11 and 5-11. Two previously unreported metabolites, 5-11 and 6-11 were also found in our studies. Several additional minor SP metabolites including 1-9 and 2-11 were also identified. A profile of the SP metabolites generated by the BBB overtime was obtained. The results from the present study provide us a better understanding of the role of blood-brain barrier in the pharmacology of SP.

Text of paper:

INVESTIGATION OF THE METABOLISM OF SUBSTANCE-P AT THE BLOOD-BRAIN BARRIER USING LC-MS/MS

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Introduction: The blood-brain barrier (BBB) protects the brain and maintains homeostasis by limiting the entry of many blood-borne molecules and peptides. The tight junctions between the endothelial cells that comprise the BBB bestow the barrier properties to BBB. Understanding the interactions between blood-borne neuropeptides such as Substance P (SP) and BBB is of utmost interest in the design of drugs for pain, and neurodegenerative diseases [1-5]. Also many neuropeptides such as SP can function as vectors for the delivery of potent drug substances to their target sites in the brain [6]. In addition, the BBB is also known to express metabolic enzymes on its surface that exhibit peptidase activity, generating metabolites which exert their own pharmacological effects. Thus investigation of the metabolism of neuropeptides such as SP at the BBB interface can assist in understanding the complex physiological effects of major neuropeptides and their metabolites.

In vitro cell culture models of BBB provide the advantage of studying the metabolism and permeation mechanisms of drug substances without the complexity associated with *in-vivo* procedures. Further, the samples generated from these *in vitro* experiments require little or no sample clean up in comparison to the *in vivo* studies. The bovine-brain microvessel endothelial cell (BBMEC) system is a well-characterized *in vitro* model of the BBB and retains many properties such as efflux, transport and metabolic pathways of intact BBB [7]. This *in vitro* model has been widely used in the investigation of peptide metabolism, transport and binding at the BBB. Various enzymes have also been identified in the BBMEC model, including the angiotensin-converting enzyme (ACE), hydolyases, and peptidases [8,9].

Substance P offers many sites in its structure for metabolic cleavage. The various enzymes that have been identified to cleave SP include ACE, aminopeptidase, neutral endopeptidase, dipeptidyl aminopeptidase, post-proline cleaving enzyme and SP degrading and hydrolyzing enzymes. ACE has been shown to be expressed in both the cytosol and the membrane surface of BBMEC. The various SP metabolites that are generated at the BBB are known to exert important physiological effects, including alteration of the levels of other neurotransmitters in the brain [10].

An earlier report of the metabolism of SP at the BBB utilized capillary electrophoresis with laser induced fluorescence detection (CE-LIF) [11]. This method involved derivitization of the ϵ amino or lysine group with naphthalene dialdehyde and cyanide and thus it is limited to detecting only lysine containing

metabolites. Although metabolism studies with SP have been performed *in vivo* using LC-MS/MS [12], its metabolism in the BBMEC model has not been investigated. Thus the objective of the present study was to investigate the metabolism of SP at the BBB and identify its major metabolites using LC-MS/MS. LC-MS/MS is capable of detecting all the metabolites of SP and is not limited to a specific functional group. The results from this study are compared with the *in vivo* studies using microdialysis sampling and with previous SP metabolism studies using CE-LIF.

Materials and Methods

Materials:

Substance P 1-11, 1-4, 1-7, 1-9, 2-11 and (Tyr⁸) 1-11 were obtained from Bachem Biosciences (King of Prussia, PA, USA). SP 3-11, 5-11 and 6-11 were acquired from Advanced ChemTech (Louisville, KY, USA). Leucine-enkephalin (Leu-Enk), CaCl₂, MgSO₄, NaCl, KCl, Na₂HPO₄, KH₂PO₄, glucose, ascorbic acid, sodium bicarbonate, endothelial cell growth factor, streptomycin, horse serum, polymycin B, amphotericin B, heparin and penicillin G were obtained from Sigma (St. Louis, MO, USA). Minimum essential media (MEM), N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) and Ham F-12 nutrient mixture (Ham's F12) were acquired from Gibco (Long Island, NY). Acetonitrile (ACN, HPLC grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and formic acid (99%) was from Acros Organics (Morris Plains, NJ, USA).

Stock Solutions:

A stock solution of 0.1 % formic acid (FA) in water was prepared diluting 1 mL of FA with 999 mL of NANOpure water (Labconco, Kansas City, MO, USA). The solution was then filtered through a 0.22 µm Magna nylon supported membrane filter from Osmonics (Minnetonka, MN, USA). The same

procedure was used to prepare a 0.1 % FA stock solution in ACN except that instead of diluting with water the FA was diluted with ACN prior to filtration. Stock solutions used for standards of SP, its metabolites and internal standard (IS) (Tyr⁸) 1-11 were made up in 0.1 % FA in water at concentrations of 100 µM for all compounds except SP 1-4, which was at 500 µM. The peptide stock solutions were stored at 4° C in 1.5 mL polypropylene microcentrifuge tubes from Fisher Scientific (Pittsburgh, PA, USA). The complete culture plating media consisted of 10 mM HEPES, 13 mM sodium bicarbonate, 100 µg/ml of streptomycin and 100 µg/ml of penicillin G in a mixture of 50 % MEM and 50 % Ham's F12. The culture media was adjusted to pH 7.26 and sterile filtered. For the plating media 55 ml of horse serum, 1.25 ml of polymyxin B (20 mg/ml) and 0.125 ml of amphotericin B were added to 500 mL of the complete culture media. The changing medium was also prepared similarly except that 1.1 ml of heparin 50mg/ml and 2.5 ml of endothelial cell growth factor were added instead of polymyxin B and amphotericin B solutions. The plating media and changing media were then stored at 4° C until use. The buffer used for metabolism experiments consisted of 10 mM HEPES in 50 % MEM and 50 % Ham's F12.

LC-MS/MS parameters

All separations were performed using a Waters 2690 HPLC system (Waters, Milford, MA, USA), which contained a refrigerated autosampler (maintained at 4° C) and a column heater unit (maintained at 25° C). Samples were placed in 250 µL volume polypropylene autosampler vials (Fisher Scientific, Pittsburgh, PA, USA) and 25 µL was injected for each sample. The samples were analyzed using a 1.0 x 50 mm Vydac C-18 analytical column with 5 µm particles (Grace Vydac, Hesperia, CA, USA). The HPLC separation of SP and its metabolites was accomplished using a flow rate of 0.2 µL/min and gradient elution. For the gradient parameters, Solvent A was 0.1 % FA in water and Solvent B was 0.1 % FA in ACN. The gradient used for the separation was: 100 % A (0.1 % FA in water) from 0 to 2 minutes, a linear ramp from 2 to 5 minutes going from 100 % A to 75 % A and 25 % B (0.1 % FA in ACN), holding at

75 % A and 25 % B from 5 to 6 minutes, a linear return from 5 to 10 minutes going from 75 % A and 25 % B to 100 % A, and a 5 minute equilibration from 10 to 15 minutes at 100 % A before the next injection. The column was connected to the HPLC system by 60 cm of 0.05" PEEK tubing (Upchurch Scientific, Oak Harbor, WA, USA) and was connected to the detector by 50 cm of 0.07" PEEK tubing (Upchurch Scientific). Tandem MS detection was accomplished using a Micromass Quattro Micro (Waters, Milford, MA, USA) in electrospray positive ionization mode (ESI+). The MS/MS settings are listed in Tables 1 and 2.

Investigation of SP metabolism in vitro using BBMECs

The BBMECs were isolated from the gray matter of bovine brains (see ref 13 for details). The BBMECs were then plated on a 12-well tissue culture plate using plating media. After 3 days, the media was replaced every alternate day until the cells were grown to reach a confluent monolayer. Metabolism studies were conducted with the BBMECs after they had attained confluency. The metabolism of SP was investigated in the BBMECs using solutions of SP (100 nM, 1 μ M or 10 μ M SP) made up in 10 mM HEPES in 50 % MEM and 50 % Ham's F12 for metabolism studies. Before the metabolism studies were initiated, the growth media in the plate was removed and the cells were rinsed with the above solution. The temperature was maintained at 37° C by placing the 12-well plate in a heated water bath. Following this, 2 mL of a SP solution was added to 9 of the 12 wells. The rest of the wells contained blank solution as a control. Substance P stability in the solution was also simultaneously investigated in a separate 24-well plate. Sixty microliter (60 μ L) aliquots were taken out over a period of 5 hours from the wells at different time intervals and diluted with 48 μ L of 0.1% FA and 12 μ L of 10 μ M IS in 0.1% FA. These samples were then stored at -20° C until analyzed by LC-MS/MS for metabolism products.

Results and Discussion

Analytical method

An electrospray LC-MS/MS method for the detection of SP and its metabolites in cell culture samples was developed. The advantages of LC-MS/MS technique over previous analytical methods for SP and its metabolites include, (i) the ability to detect compounds based on mass-to-charge (m/z) ratio, (ii) no need for sample derivatization (fluorescence and immuno assay based methods), (iii) does not generate radioactive waste, (iv) short chromatographic runs, (v) amenable to automation, and (vi) excellent sensitivity and selectivity. Although other mass spectrometric techniques (such as MALDI) can be used for peptide identification, LC-MS/MS remains the choice for quantitative analysis of peptides [14]. Electrospray ionization in the positive mode was selected as the method for the detection of SP and its metabolites. The hydrophilic nature of the peptide and the presence of ionic residues in SP structure make the compound readily ionizable under ESI⁺ conditions, thus improving the sensitivity of the assay. The mobile phase for the analysis is composed of an acidic aqueous phase (0.1% formic acid) with an organic modifier (acetonitrile).

Using this LC-MS/MS method, with (Tyr⁸)-SP as internal standard, chromatographic separation of SP and its fragments resulted in well resolved peaks without any issues of co-elution and cross-talk among the compounds (Fig. 1). The linearity and linear range was assessed for SP and each of the metabolites (see Table 3 for results). The linearity (r^2) was greater than 0.97 for all 8 compounds and with the exception of SP 1-4; the linear range was from 1 or 2 nM to at least 500 nM. The LC-MS/MS was also a very sensitive assay with a LOD of 2 nM or less for all compounds except SP 1-4 (Table 3). The lack of linear range and sensitivity for SP 1-4 is likely due to ion suppression by co-eluting salts from the cell media. The final method required minimal sample preparation (only dilution and addition of internal standard), and no derivatization. The MS based method could also be automated and had a

separation time of less than 10 minutes and a 15-minute total analysis time (5 min for column equilibration between runs).

SP metabolism with BBMECs

Prior to the investigation of metabolism of SP by BBMEC, the use of buffer solution as a satisfactory medium for conducting the metabolism studies was confirmed. The stability of SP in the buffer solution without the presence of cells was determined by storing it in a polypropylene tube at 4° C for 48hrs (Fig. 2a) and at 37° C for 24hrs (Fig. 2b). The solutions showed no signs of degradation. Figure 2c shows a TIC chromatogram obtained after 5 hours from a well containing cells, but no SP. The only peak observed is that of the internal standard which was added to the sample prior to injection. Figure 2d shows a TIC chromatogram at the 5 hour time point of a well at 37° C with no cells, but containing 10 µM SP. In this figure, a trace amount of degradation of SP was detected (3-11); however, this degradation was less than 0.1 % of the SP peak and therefore was not likely to cause a problem with metabolite quantitation. No peaks were observed in the wells containing the buffer solution without any cells. Chromatograms obtained from the incubation of 10 µM SP with BBMECs for 5 hrs are shown in Fig. 3 (low intensity metabolites) and Fig. 4 (high intensity metabolites). The concentration-time profile for formation of SP metabolites over the 5-hour period is shown in Fig. 5. Substance P 3-11 and 1-7 were the major metabolites detected. Smaller amounts of SP 1-4, 1-7, 1-9, 2-11 and 5-11 were also found. After 5 hours, 70 % of SP was found to remain intact and unmetabolized in the buffer.

The metabolism of SP was also investigated at lower concentrations (1 µM and 100 nM) to determine if the metabolite profile changes with concentration. These concentrations were closer to those observed physiologically (Fig. 6 and 7). The major metabolite generated at high concentrations (1 µM and 100 nM) was SP 3-11; however, at 100 nM SP, the concentration of SP 3-11 generated was

similar in magnitude to that of SP 5-11 and SP 6-11. Only 3 metabolites could be detected from the incubation at 100 nM, due to the sensitivity limit of the LC-MS/MS method. However at 1 μ M SP, all the metabolites except 1-4 could be detected and quantified.

Metabolism comparison with the in vivo study and previous in vitro studies

The *in vitro* results described here indicate that SP 3-11 is the major metabolite formed at the BBB and that SP 2-11, while detected, occurs at a much lower concentration than any of the other metabolites monitored. These results were compared to previous *in vivo* metabolism studies [15-18]. *In vivo* brain microdialysis studies conducted in our lab using LC-MS/MS have shown that SP 1-7 and SP 3-11 were the major metabolites in the striatum. In addition, SP 2-11 was observed as a minor metabolite in these studies [17]. These results correlate very well with those observed *in vitro* using the BBMECs; SP 3-11 and SP 1-7, at 10 μ M SP, were the major metabolites and SP 2-11 was the metabolite that occurred at the lowest concentration.

Previous work in our lab on SP metabolism using BBMECs and CE-LIF detection also found SP 3-11 as the major metabolite [11]. Other groups have reported SP 5-11 and SP 6-11 [12] and SP 1-7 and SP 1-4 [16] as the major metabolites of SP *in vivo*. Our laboratory is the only group to report the appearance of SP 2-11 as a metabolite both *in vivo* with rats or *in vitro* with the BBMEC cell culture model. Because SP 2-11 occurs at very low concentrations (nM), a very sensitive analytical method was necessary for its detection.

Enzymatic cleavages and physiological importance of metabolites

Many of the metabolites of SP (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂) have been shown to exhibit important physiological effects. The major metabolite detected in the LC-MS/MS experiments, (SP 3-11) results from the cleavage of SP by endopeptidase EC 3.4.21.26, a post-proline cleaving enzyme [20, 21]. This metabolite has been shown to cause a SP-like caudally-directed biting and scratching behavior when administered to mice [22]. The SP metabolite 1-7 is generated by endopeptidase 24.11 [23-25] and also by angiotensin converting enzyme (ACE) [26, 27]. This metabolite has been shown to enhance dopamine release, to increase the concentration of dihydroxyphenylacetic acid in the brain [28] and to down-regulate NO synthesis [29].

Two of the metabolites that were observed in these studies (SP 1-4 and SP 5-11) are formed simultaneously by two different enzymes, endopeptidase EC 3.4.21.26 [18, 19] and SP-degrading endopeptidase [30]. Both enzymes cleave SP at the Gln⁵-Gln⁶ bond. Metabolite 1-4 has been shown to significantly increase dopamine outflow [31] while SP 5-11 increases the release of Asp, Glu, Asn, Gly, and taurine [32]. Matrix metalloproteinase-9 has been shown to cleave SP to produce SP 1-9 [33]. As with metabolite SP 1-4, SP 1-9 has been determined to significantly increase dopamine outflow [34].

The Substance P metabolite 6-11 is formed by both SP-degrading endopeptidase [30] and matrix metalloproteinase-9 [33]. This metabolite was demonstrated to stimulate an 8-fold increase in NK-1R phosphorylation [35] and has also been shown to decrease striatal dopamine release [36]. While the metabolite SP 2-11 has been shown to be produced by the hydrolysis of the Arg¹-Pro² bond with aminopeptidase P [37] there have been no reports to date of its biological activity.

These results presented here, when taken in conjunction with similar results obtained from *in-vivo* studies, indicate that BBB and other endothelial tissues constitute the major sites for the metabolism of SP released in the periphery and thus play an important role in the pharmacological and toxic effects of SP metabolites.

Conclusion:

The results of SP metabolism at the BBB from this study correlate well with *in vivo* results obtained using LC-MS/MS analysis as well as with previous work using BBMECs. Two previously unreported metabolites; SP 5-11 and SP 6-11 were found to be produced and were detected at physiologically relevant concentrations. This present study also demonstrates that BBMECs can potentially be used to investigate the metabolism of neuropeptides at the BBB, without the need for complex *in vivo* experimental procedures.

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Legends for Figures:

Table 1: Precursor ion m/z's, product ion m/z's, cone voltages and collision energy values for SP, its metabolites and internal standard.

Table 2: Optimal parameters for SP and metabolites which are held constant for each individual compound.

Table 3: Linearity, linear range and LOD for SP and metabolites in cell media.

Figure 1: Chromatogram of 1 μ M SP, metabolites and IS in 50% cell media. Separation was performed using a gradient of 100 % A (0.1 % FA in water) from 0 to 2 minutes, a linear ramp from 2 to 5 minutes going from 100 % A to 75 % A and 25 % B (0.1 % FA in ACN), holding at 75 % A and 25 % B from 5 to 6 minutes, a linear return from 5 to 10 minutes going from 75 % A and 25 % B to 100 % A

Figure 2a: TIC of SP, metabolites and IS showing the stability of 5 μ M SP in cell experimental media after 2 days at 4° C.

Figure 2b: TIC showing stability of 5 μ M SP in cell experimental media after 24 hours at 37° C (no IS added to this sample).

Figure 2c: TIC chromatogram of well with cells and blank bank buffer (no SP) after 5 hours.

Figure 2d: TIC chromatogram of well with a solution of 10 μ M SP with no cells after 5 hours.

Figure 3: Chromatogram of low intensity metabolites after 10 μ M SP has been incubated in wells with BBMECs for 5 hours; combined traces of 1-4, 1-7, 1-9, 2-11 and 5-11 are shown.

Figure 4: Chromatogram of high intensity metabolites after 10 μ M SP has been incubated in wells with BBMECs for 5 hours; combined traces of IS, 3-11 and 6-11 are shown.

Figure 5: Time profile for metabolism of 10 μ M SP in BBMECs (n = 4).

Figure 6: Time profile for metabolism of 1 μ M SP in BBMECs (n = 4).

Figure 7: Time profile for metabolism of 100 nM SP in BBMECs (n = 4).

Table 1:

Compound	Molecular weight	Precursor m/z	Product m/z	Cone Voltage	Collision Energy
1-4	497	249.2	70.0	18	30
1-7	900	450.7	70.1	30	40
1-9	1104	552.9	120.2	30	40
1-11	1347	674.6	120.2	35	45
(Tyr ⁸) 1-11	1364	682.6	104.2	35	40
2-11	1192	596.4	104.2	25	25
3-11	1094	547.9	104.2	20	20
5-11	869	869.4	120.2	40	60
6-11	741	741.4	120.2	35	55

Table 2:

MS/MS tune parameter	Parameter setting	Parameter unit (N/ A if unit less)
Capillary voltage	2.5	kV
Extractor	2	V
RF lens	0	N/A
Source temperature	100	°C
Desolvation temperature	350	°C
Desolvation gas flow	675	L/hr
Cone gas flow	40	L/hr
LM and HM 1 resolution	12	N/A
Ion energy 1	0.5	N/A
Entrance	2	N/A
Exit	1	N/A
LM and HM 2 resolution	15	N/A
Ion energy 2	2	N/A
Multiplier voltage	650	V
Gas cell pirani pressure	3.6 e ⁻³ (argon)	mbar

Table 3:

Compound	Linear Range (nM) in 50% cell media	Linearity (R^2) in 50% cell media	LOD (nM) in 50% cell media
1-4	50-1000	0.990	50
1-7	2-500	0.974	2
1-9	1-1000	0.990	1
1-11	1-500	0.983	1
2-11	1-500	0.985	< 1
3-11	1-500	0.990	< 1
5-11	1-500	0.979	1
6-11	1-500	0.989	1

Figure 1

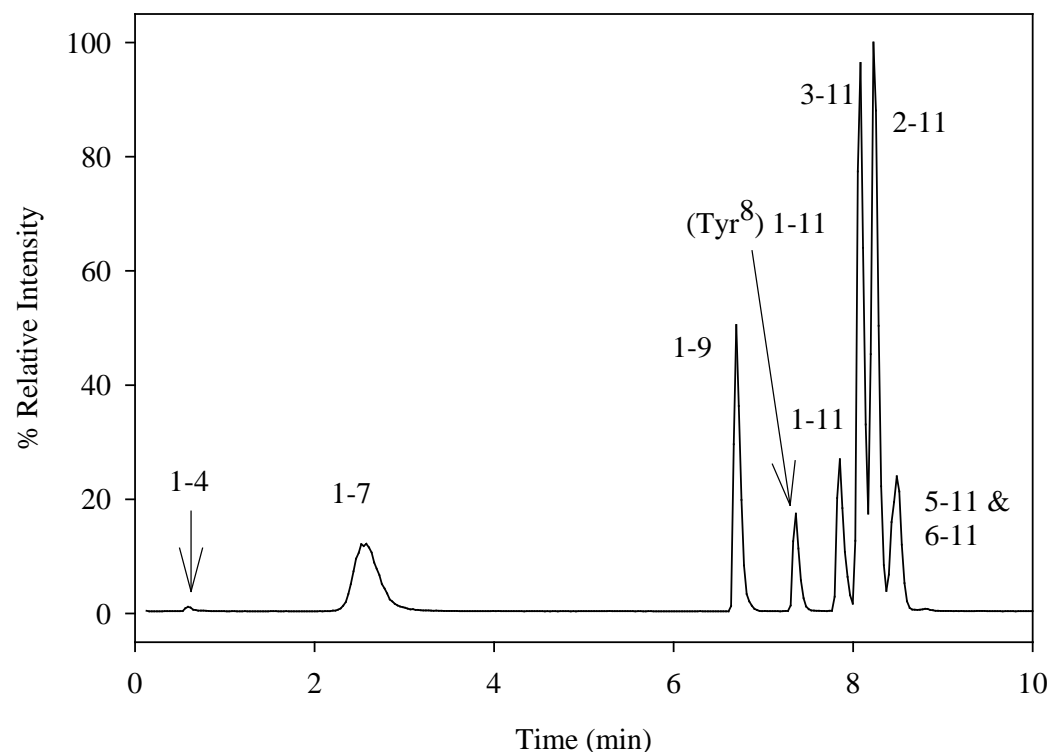


Figure 2a:

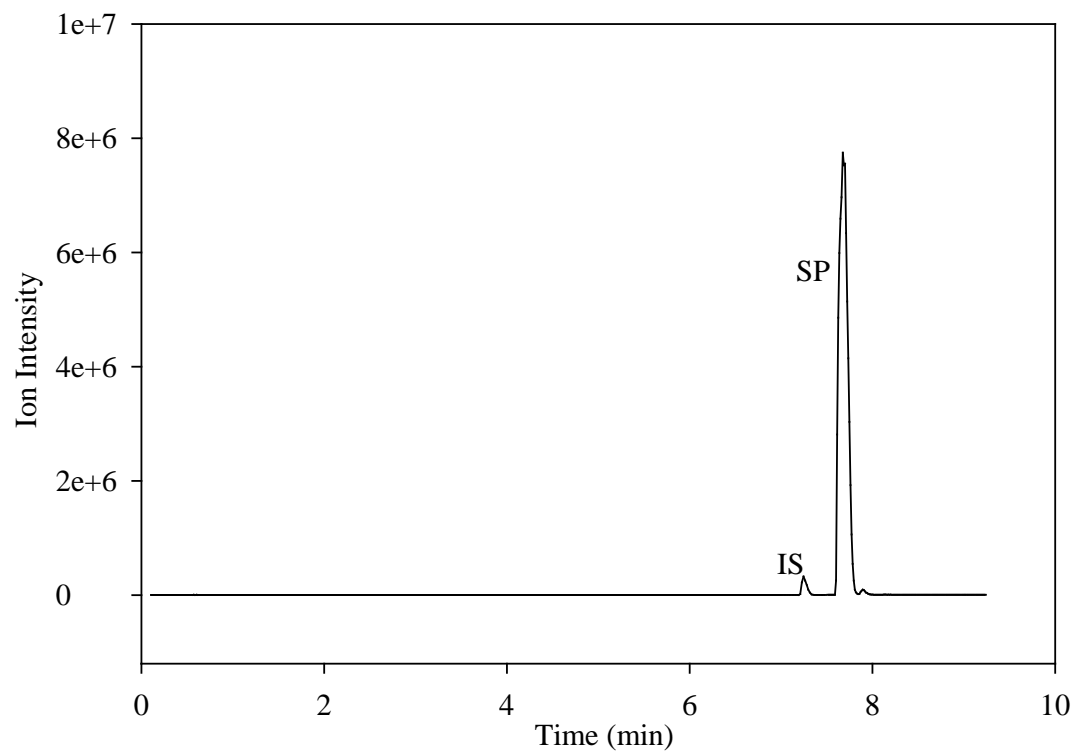


Figure 2b

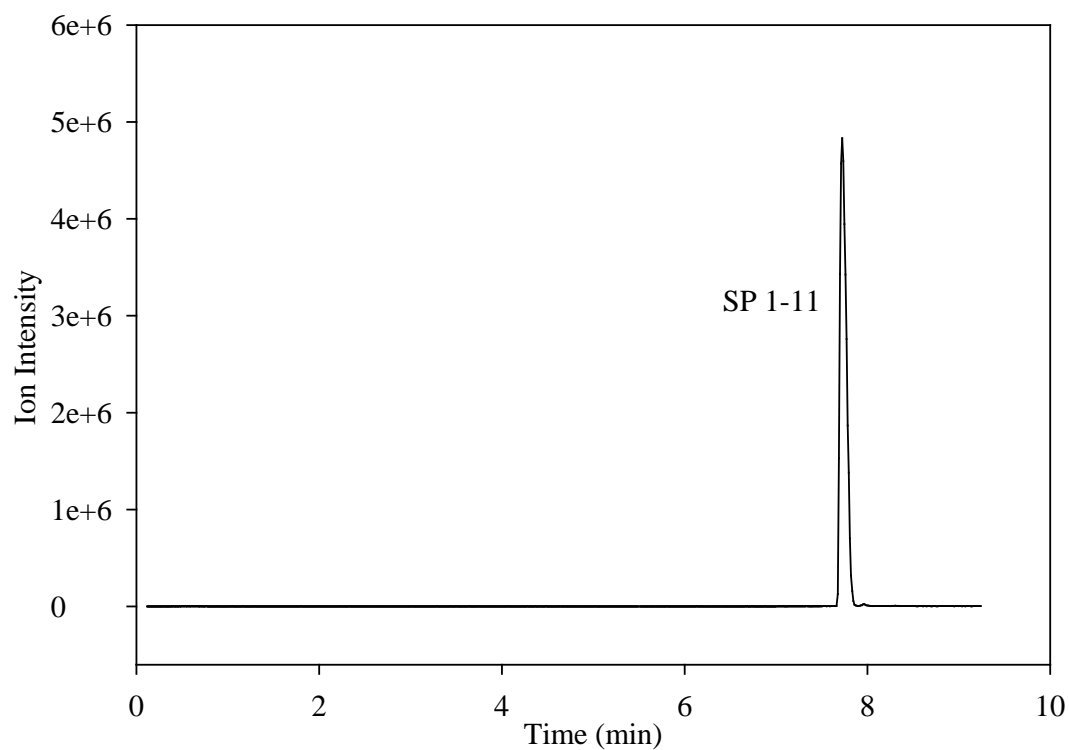


Figure 2c

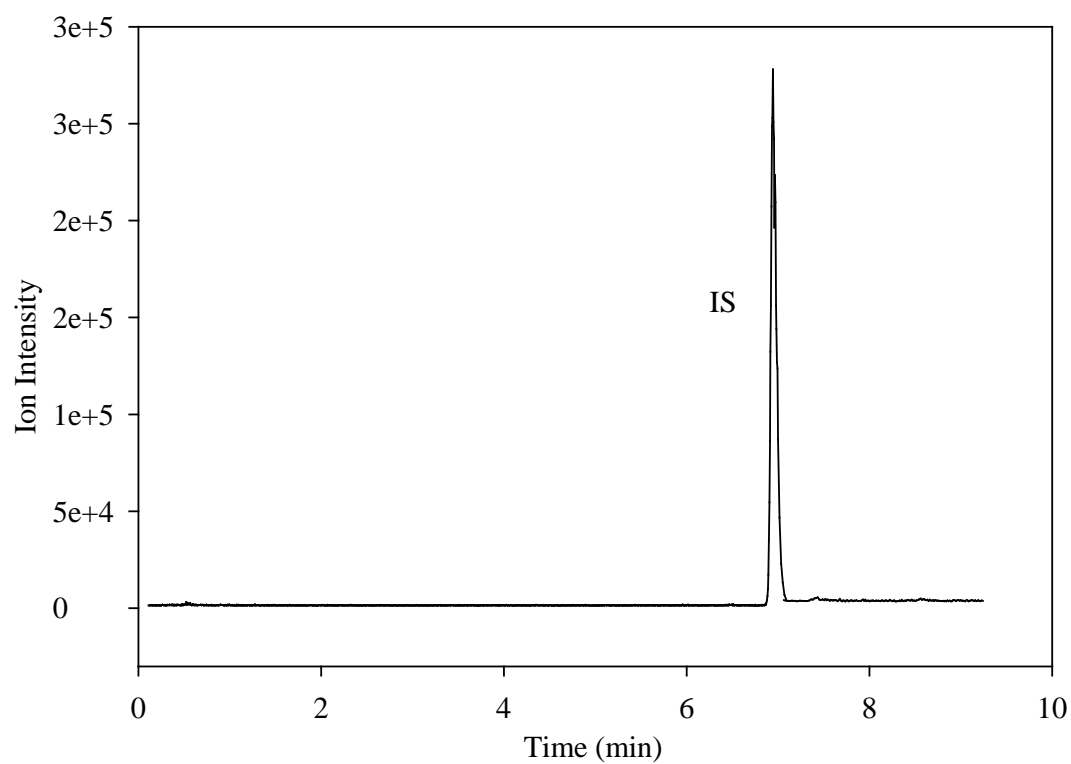


Figure 2d

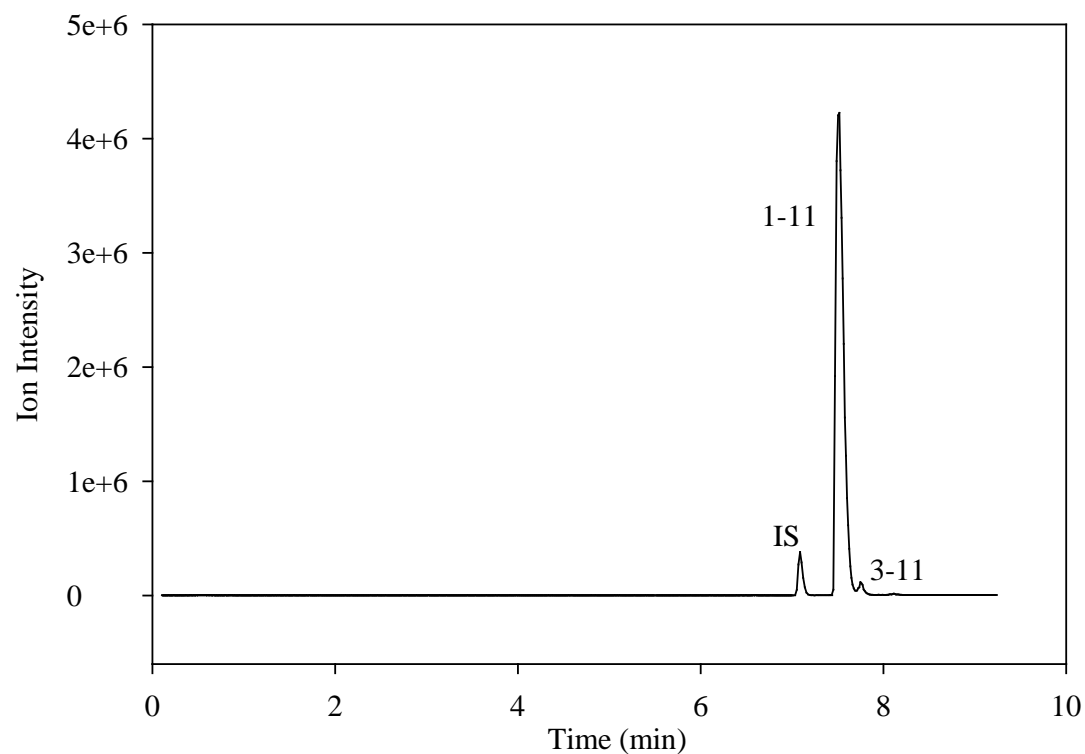


Figure 3

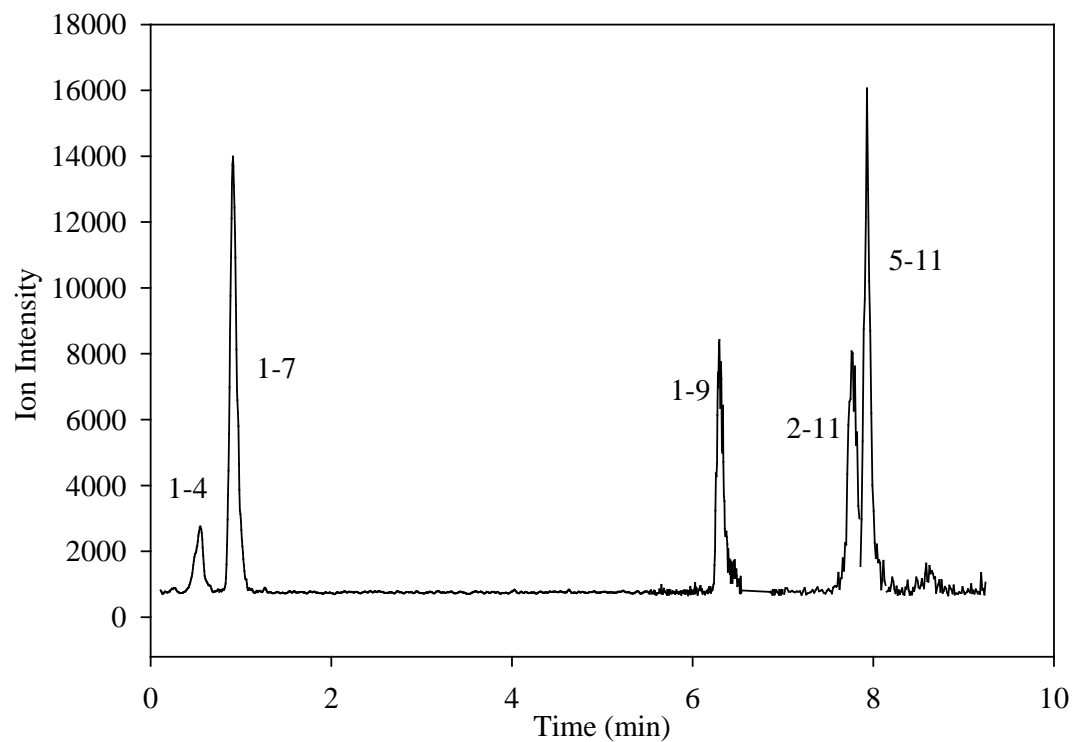


Figure 4

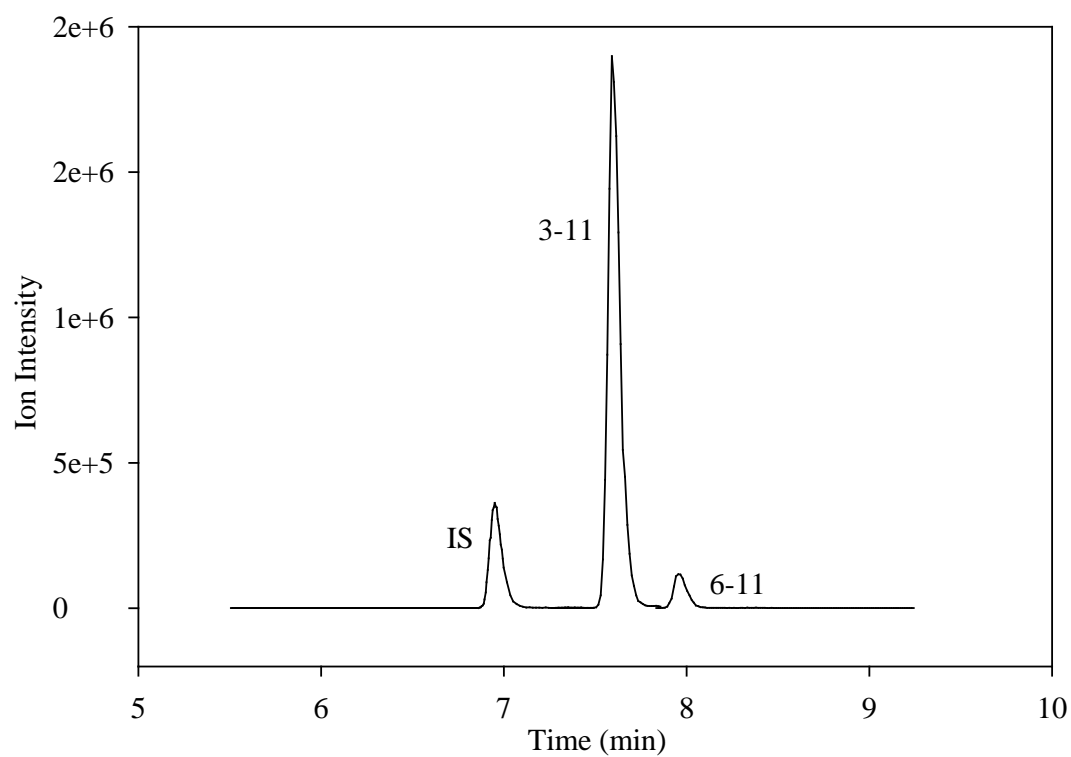


Figure 5

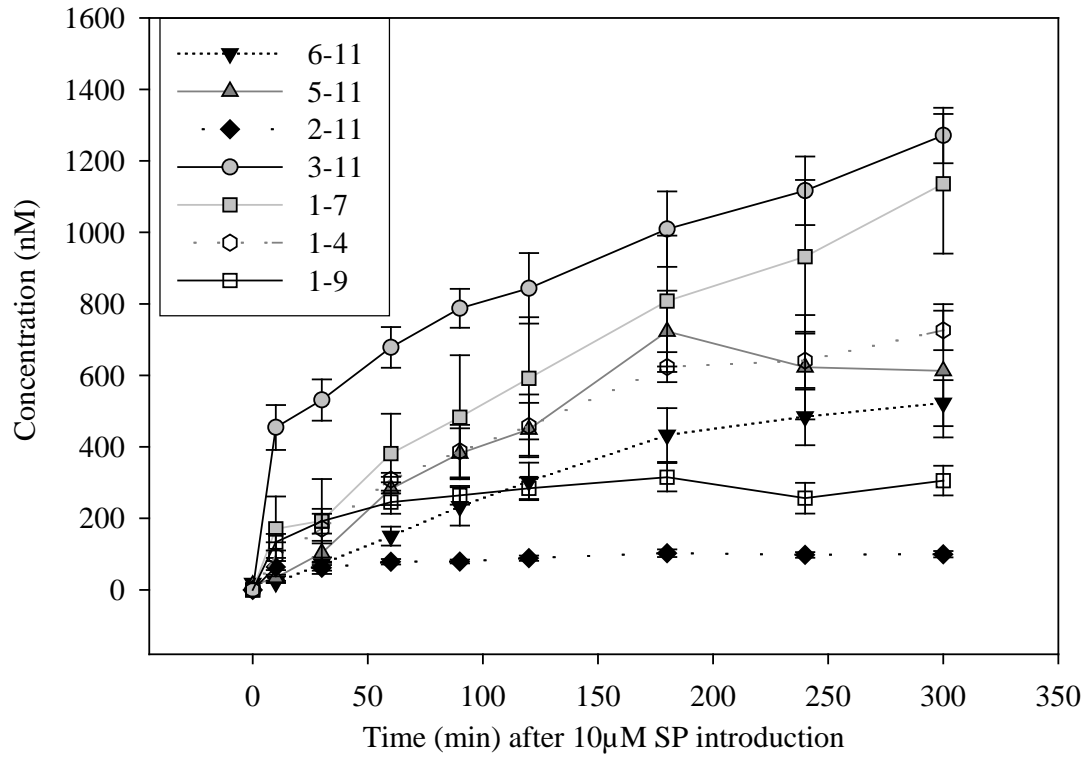


Figure 6

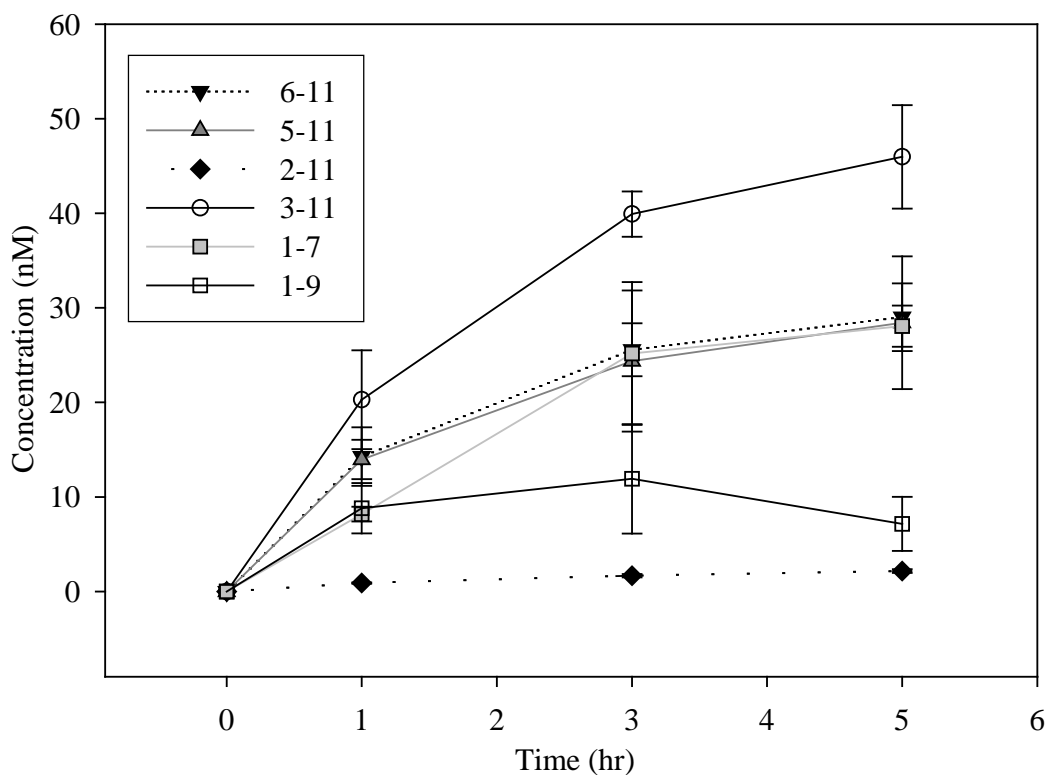


Figure 7

